PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of:

Pellett, et al.

Serial No. **08/480,850**

Filed: **June 7, 1995**

For: **NOVEL BACULOVIRUS EXPRESSION VECTORS AND RECOMBINANT ANTIGENS FOR DETECTING TYPE SPECIFIC ANTIBODIES TO HERPES SIMPLEX VIRUS**

Art Unit: **1643**

Examiner: **Lee, D.**

APPELLANTS' BRIEF ON APPEAL

Assistant Commissioner for Patents
Washington, DC 20231

To the Honorable Board of Patent and Appeals and Interferences:

The Appellants hereby submit their Brief on Appeal in the patent application identified above. The fee of $300.00 required by 37 C.F.R. § 1.17(c) for the submission of an appeal brief is enclosed herewith.

Real Party in Interest

The real party in interest is United States of America, as represented by the Secretary, Department of Health and Human Services, Washington, D.C., as assignee of the pending application.

Related Appeals and Interferences

Appellants are aware of two related applications that are also currently under appeal. United States Serial No. 07/691,728, of which the present application is a divisional, has been submitted on appeal to the board since June 20, 1994. United States

Serial No. 08/472,507, which is also a divisional of the 07/691,728 application, is also currently on appeal. While the technologies of the three applications are related and the three appeals may share some underlying issues, Appellants do not believe that the three appeals necessarily share the same ultimate issue of patentability.

Status of Claims

Claims 7, 8, and 16-21 are pending in this case and currently stand rejected. Claim 4 was cancelled in the Preliminary Amendment filed June 7, 1995. Claim 16 was added in the Amendment filed July 30, 1996. Claims 17-21 were added in the Amendment filed October 28, 1997.

Status of Amendments

No amendment was filed after final rejection.

Summary of Invention

The present invention is directed to novel herpes simplex virus (HSV) gG-1 and gG-2 antigens. The antigens of the present invention are recombinant HSV glycoproteins and are best described by the process used to produce them. These antigens are produced by an expression system using novel recombinant baculoviruses (*Autographa californica*). The gene for either HSV gG-1 or gG-2 glycoproteins is inserted into the novel vectors such that the translation initiation codon of the HSV glycoprotein gene directly abutts the polyhedrin promoter sequence of the native baculovirus. Thus, the gG-1 or gG-2 gene is juxtaposed with the baculovirus polyhedrin gene precisely at the translation initiation cite without missing any nucleotide present in the 5' region of the wild-type polyhedrin gene or without adding any extraneous nucleotides at the translation initiation cite. The recombinant baculoviruses containing the gG-1 or gG-2 gene is then used to infect insect cells which, in turn, produce an antigen, the HSV glycoprotein corresponding to the gene contained in the baculovirus vector.

Because recombinant baculoviruses thus constructed have much higher rates of expression, glycoprotein antigens produced by this process can be isolated in much

purer form. Additionally, antigens produced by the process are structurally different from antigens produced by other means, including other recombinant baculoviruses.

Issues

I. Whether recombinant HSV gG-1 and gG-2 antigens are patentable over Lee, et al. (AA1 - *J. Clin. Microbiology*, 1985) or Lee, et al. (AW - *J. Virological Methods*, 1986) in view of Luckow, et al. (AO - *Bio/Technology*, 1988), Matsuura, et al. (AP - *J. Gen. Virology*, 1987), and further in view of Krishna, et al. (*J. Gen. Virology*, 1989).

II. Whether recombinant HSV gG-1 and gG-2 antigens produced by employing a recombinant baculovirus having the 5' nontranslated leader sequence of the polyhedrin gene joined to the coding region of a foreign gene precisely at the translation initiation codon of the polyhedrin gene, without either missing any nucleotide present in said initiation codon or introducing any extraneous nucleotide at the intiation codon site are patentable over Lee, et al. (AA1) or Lee, et al. (AW) in view of Luckow, et al. (AO), Matsuura, et al. (AP), and further in view of Krishna, et al. (*J. Gen. Virology*, 1989).

Grouping of Claims

The Appellants contend that not all of the rejected claims stand or fall together, but the following groups are each separately patentable in view of the art relied upon by the Examiner and the evidence submitted by the Appellants.

Group	Independent Claim	Dependent Claims
Group I	Claim 16	Claims 18 & 19
Group II	Claims 7 & 8	Claim 17*, 20 & 21

*Claim 17 depends from Claim 16 but is separately patentable from it.

Argument

I. Separable Patentability of the Claims of Group I and Group II

Pursuant to 37 C.F.R. § 1.192(c)(7), Appellants include the following explanation of the separable patentability of the claims of Group I and Group II. The claims of Group II contain the limitations of the claims of Group I plus an additional limitation not contained by the claims of Group I. Specifically, the claims of Group II contain a product-by-process limitation that recites that the claimed antigens are produced by employing a recombinant baculovirus having the 5' nontranslated leader sequence of the polyhedrin gene joined to the coding region of a foreign gene precisely at the translation initiation codon of the polyhedrin gene, without either missing any nucleotide present in said initiation codon or introducing any extraneous nucleotide at the initiation codon site. Additional evidence has been submitted by Appellants during prosecution to show that antigens produced by the process recited in the product-by-process limitation are patentably distinct and unobvious. The product-by-process limitation and the additional submitted evidence give Group II separable patentability with respect to Group I.

II. Summary of Argument

The Examiner rejected claims 7, 8 and 16-21 under 35 U.S.C. § 103 as being obvious and unpatentable over Lee, et al. (AA1) or Lee, et al. (AW) in view of Luckow, et al. (AO), Matsuura, et al. (AP), and further in view of Krishna, et al. (*J. Gen. Virology*, 1989). The Examiner asserted that the cited references, in combination, disclose all of the limitations of the claimed invention.

The claims of Group I (Claims 16, 18 and 19) and the claims of Group II (Claims 7, 8, 17, 20 and 21) are drawn to recombinant baculovirus expressed HSV gG-1 and gG-2 antigens. The Examiner has cited references that disclose non-recombinant HSV gG-1 and gG-2 antigens and references that disclose the use of various baculovirus vectors to express proteins. However, Appellants have demonstrated that recombinant HSV gG-1

and gG-2 antigens are distinct from non-recombinant HSV gG-1 and gG-2 antigens and that the cited references provide no motivation for producing pure recombinant HSV gG-1 and gG-2 antigens in baculovirus vectors.

Furthermore, the claims of Group II are unobvious because they contain a product-by-process limitation. Product-by-process claims are patentable if the product of the process recited in the product-by-process limitation is patentably distinct from prior art products. Ex parte Gray, 10 USPQ 2d 1922 (Bd. Of Patent Appeals and Interferences, 1989).

Appellants understand that the burden is on the Appellants to present evidence that the product identified by the product-by-process claims exhibits unexpected properties not exhibited by the prior art product. Appellants have met this burden by submitting evidence that the gG-1 and gG-2 antigens produced by the baculovirus vector recited in the product-by-process limitation of Group II are patentably distinct from gG-1 and gG-2 antigens that would be produced by other putative baculovirus vectors.

III. The cited references fail to make the baculovirus expressed recombinant HSV gG-1 and gG-2 antigens of Groups I and II obvious.

The Examiner rejected claims 7, 8 and 16-21 under 35 U.S.C. § 103 as being obvious and unpatentable over Lee, et al. (AA1) or Lee, et al. (AW) in view of Luckow, et al. (AO), Matsuura, et al. (AP), and further in view of Krishna, et al. (*J. Gen. Virology*, 1989). However, there is no teaching in any of the cited references of recombinant gG-1 or gG-2 antigens produced by any means, and there is no motivation in any of the cited references to combine the teachings of the references in order to create the recombinant antigens of the present invention.

Lee et al., *J. Clin. Microbiol.* 22:641-644 (1985), teach the use of purified herpes simplex virus type 2-specific glycoprotein (gG-2) in an immunodot enzymatic assay for the detection of HSV-2 antibodies in human serum. The gG-2 antigen used by Lee *et*

al. (1985) was purified from HSV-2-infected HEp-2 cells using immunoaffinity chromatography columns containing anti-gG-2 mouse monoclonal antibodies. Lee et al. (1985) fail to disclose a recombinant gG-2 antigen.

Lee et al., *J. Virol. Methods* 14:111-118 (1986), teach the purification of herpes simplex virus type 1-specific glycoprotein (gG-1) using a mouse monoclonal antibody. Lee et al. (1986) fail to disclose a recombinant gG-1 antigen.

Luckow et al., *Bio/Technology* 6:47-55 (1988), is a review article directed to baculovirus expression vectors. Luckow et al. describe numerous factors affecting the expression of foreign genes by baculovirus vectors. Luckow et al. fail to teach the expression of HSV gG-1 or gG-2 antigens in baculovirus vectors and fail to suggest that HSV gG-1 and gG-2 antigens be expressed in baculovirus vectors.

Matsuura et al., *J. Gen. Virol.* 68:1233-1250 (1987), teach that the polyhedrin gene promoter of *Autographa californica* nuclear polyhedrosis virus (AcNPV) is useful for high level expression of a glycoprotein of lymphocytic choriomeningitis virus. Matsuura et al. fail to teach the expression of HSV gG-1 or gG-2 antigens in baculovirus vectors and fail to suggest that HSV gG-1 and gG-2 antigens be expressed in baculovirus vectors.

The Examiner has provided no reference teaching the production of recombinant HSV gG-1 and gG-2 antigens using any vector.

The claimed recombinant gG-1 and gG-2 antigens and naturally produced gG-1 and gG-2 antigens, such as those disclosed by Lee et al. (1985) and Lee et al. (1986), are not equivalent. Recombinant HSV gG-1 and gG-2 antigens are structurally different from gG-1 and gG-2 antigens produced naturally by the herpes simplex virus types 1 and 2. As explained in Appellants' specification, the recombinant proteins are different in that they are differently glycosylated than the non-recombinantly produced glycoproteins. (See page 25, line 20 to page 26, line 7 of the present specification.) Therefore, by describing the gG-1 and gG-2 antigens as "recombinant" antigens, they are

by definition structurally different from the naturally-produced glycoprotein antigens described by Lee et al. (1985) and Lee et al. (1986).

Structural differences between the claimed recombinant HSV gG-1 and gG-2 antigens and naturally produced gG-1 and gG-2 antigens are shown in Figures 3 and 4 of the scientific article by Sanchez-Martinez and Pellett, "Expression of HSV-1 and HSV-2 Glycoprotein G in Insect Cells by Using a Novel Baculovirus Expression Vector," *Virol.* 182:229-238 (1991) which was submitted by the Appellants during the prosecution of the present application. This article describes experimental data generated by the Appellants that was included in Figures 3 and 4 and on page 21, lines 3-16 and page 23, lines 19-30 of the present specification. For example, Figure 3C of the Sanchez-Martinez and Pellett article provides an immunoblot analysis comparing the molecular weights of proteins extracted from Sf9 cells infected with the baculovirus vector AcDSMgG-1 containing the gG-1 gene (recombinant gG-1) with proteins extracted from HEp-2 cells infected with HSV-1 (non-recombinant gG-1). The recombinant gG-1 shows strong bands at 42 and 43 kDa, whereas the non-recombinant gG-1 shows a smear between 50 and 57 kDa. Similarly, Figure 4D of the Sanchez-Martinez and Pellett article provides an immunoblot analysis comparing the molecular weights of proteins extracted from Sf9 cells infected with the baculovirus vector AcDSMgG-2 containing the gG-2 gene (recombinant gG-2) with proteins extracted from HEp-2 cells infected with HSV-2 (non-recombinant gG-2). The recombinant gG-2 shows distinct bands at 107, 118, 128 and 143 kDa, whereas the non-recombinant gG-2 shows a smear between 78 and 118 kDa. It is well understood by those skilled in the art that proteins having different immunoblot band patterns are considered structurally different.

Furthermore, the claimed recombinant gG-1 and gG-2 antigens are produced in the absence of the other 80 herpes simplex virus proteins that are produced naturally by herpes simplex virus type 1 or herpes simplex virus type 2-infected mammalian cells. Nearly every gene encoded by herpes simplex virus type 1 has a genetic

counterpart in herpes simplex virus type 2, and the protein products of these homologous genes have significant antigenic cross-reactivity, except for gG-1 and gG-2. This is the basis of the unique utility of these proteins for use as antigens in the construction of HSV type-specific serologic assays. The claimed antigens are therefore much purer and less likely to be contaminated by HSV-type cross-reactive antigens than gG-1 and gG-2 proteins produced by other means. Appellants submit that this purity is not just a matter of degree but rather one of kind. The ability to produce these type-specific HSV antigens in the complete absence of other non-type-specific HSV antigens gives the claimed antigens a new level of utility in the construction of HSV type-specific assays.

In addition, the ability to produce these type-specific HSV antigens in the complete absence of other non-type-specific HSV antigens allows for greater quantities of the desired gG-1 and gG-2 antigens to be produced without any of the highly undesirable HSV proteins being produced. Thus, the production of these type-specific HSV antigens is much safer.

Appellants have shown that the recombinant HSV gG-1 and gG-2 antigens are both purer and structurally different from the naturally produced HSV gG-1 and gG-2 antigens of the prior art. Furthermore, Appellants have shown that, while Luckow et al. and Matsuura et al. disclose general advantages of baculovirus expression systems, none of the cited references contain a suggestion that recombinant HSV gG-1 and gG-2 be produced with the baculovirus expression system. Specifically, the references do not disclose the specific utility and safety advantages of producing type-specific HSV antigens in the absence of the multitude of non-type-specific HSV antigens. Therefore, Appellants submit that the claims of Group I and Group II are unobvious over the cited art by virtue of the limitation "recombinant."

IV. The gG-1 and gG-2 antigens of Group II, which are produced by use of the novel baculovirus, are patentably distinct from gG-1 or gG-2 antigens produced by use of other putative baculoviruses and unobvious over the cited references.

A. The recombinant gG-1 and gG-2 antigens defined by the product-by-process limitation included in the claims of Group II are patentably distinct from gG-1 or gG-2 antigens produced by use of other putative baculoviruses.

The Examiner has cited art to argue that it would have been *prima facie* obvious for a skilled artisan to produce the HSV gG-1 or gG-2 antigens in a baculovirus expression system. Specifically, the Examiner has argued that it would have been obvious to one skilled in the art to produce the gG-1 and gG-2 proteins of Lee, et al. in the baculovirus expresion systems of Luckow, et al. and Matsuura, et al in further view of Krishna et al.

Lee et al., *J. Clin. Microbiol.* 22:641-644 (1985), teach the use of purified herpes simplex virus type 2-specific glycoprotein (gG-2) in an immunodot enzymatic assay for the detection of HSV-2 antibodies in human serum. The gG-2 antigen used by Lee et al. (1985) was purified from HSV-2-infected HEp-2 cells using immunoaffinity chromatography columns containing the anti-gG-2 mouse monoclonal antibodies H966 and H1206. Appellants respectfully submit that Lee et al. (1985) fail to disclose a recombinant gG-2 antigen.

Lee et al., *J. Virol. Methods* 14:111-118 (1986), teach the purification of herpes simplex virus type 1-specific glycoprotein (gG-1) using the mouse monoclonal antibody H1379-2. Appellants respectfully submit that Lee et al. (1986) fail to disclose a recombinant gG-1 antigen.

Luckow et al., *Bio/Technology* 6:47-55 (1988), is a review article directed to baculovirus expression vectors. Luckow et al. describe numerous factors affecting the expression of foreign genes by baculovirus vectors including optimizing placement of the

foreign gene within the transfer vector. Appellants respectfully submit that Luckow et al. fail to teach use of the vector pAcDSM, fail to teach insertion of the foreign gene precisely at the translation initiation codon of the polyhedrin gene, without either missing any nucleotide present in said initiation codon or introducing any extraneous nucleotide at the initiation codon site, and fail to teach or suggest expression of herpes simplex virus antigens.

Matsuura et al., *J. Gen. Virol.* 68:1233-1250 (1987), teach that the polyhedrin gene promoter of *Autographa californica* nuclear polyhedrosis virus (AcNPV) is useful for high level expression of a glycoprotein of lymphocytic choriomeningitis virus. Matsuura et al. suggest that the synthesis is related to the integrity of the 5' non-coding region of the polyhedrin gene.

In response, Appellants submitted evidence that their claimed proteins did, in fact, exhibit unexpected characteristics when compared to the gG-1 and gG-2 proteins that the Examiner argued were made obvious by the cited art. Specifically, Appellants provided the Declaration under 37 C.F.R. § 1.132 of Philip E. Pellett, a co-inventor of the present application. The Declaration states that Dr. Pellett and his colleagues designed several experiments to demonstrate differences between the claimed herpesvirus glycoprotein gG-1 produced using the novel recombinant baculovirus vector AcDSMgG-1 and the herpesvirus glycoprotein gG-1 produced using the baculovirus vector Ac373'gG-1. The results of these experiments are best shown in the data published in the scientific article entitled "Expression of HSV-1 and HSV-2 Glycoprotein G in Insect Cells by Using a Novel Baculovirus Expression Vector," authored by Demetrio Sanchez-Martinez in *Virology* 182: 229-238 (1991), a copy of which was submitted during prosecution and is attached to this Brief in the Appendix.

The novel baculovirus vector AcDSMgG-1 was constructed by effectively removing a nucleotide region from the pAcDSM transfer vector (between the *Pst*I and *Hind*III restriction sites) and inserting in its place a synthetic oligomer followed by the

engineered gene as shown in Figure 1, Panel B of the *Virology* paper. As shown in Figure 1, Panel C of the *Virology* paper (page 231), in the novel AcDSMgG-1 vector, the nucleotide sequence 5' to the translation initiation codon is identical to and aligns with that of wild type AcNPV (polyhedrin), whereas the Ac373'gG-1 vector includes 21 extraneous nucleotides. By constructing a vector in this way, the novel baculovirus transfer vector pAcDSM joins the herpes simplex virus type 1 glycoprotein gene (or type 2 glycoprotein gene) precisely at the translation initiation codon of the polyhedrin gene.

This evidence of patentable distinction proffered by the Appellants to show that the claimed product is patentable over other proteins produced in baculovirus expression systems can be classified into two categories. First, Appellants have presented evidence to demonstrate that the claimed product can be isolated in pure form due to the higher expression of the process used to produce the claimed product. Second, Appellants have submitted evidence to demonstrate structural differences between the claimed product and proteins generated by other baculovirus expression systems.

1. The claimed gG-1 and gG-2 antigens defined by the product-by-process limitation are purer than gG-1 and gG-2 antigens produced by other putative baculovirus vectors.

As discussed above, the claims of Group II are directed to recombinant HSV gG-1 and gG-2 antigens produced from a novel baculovirus vector. The claimed recombinant antigens are produced in greater quantities thereby yielding a purer product than recombinant gG-1 and gG-2 antigens produced using alternative baculovirus systems such as the pAc373 baculovirus system.

Panel B of Figure 2 is a photograph of a slot-blot analysis of serial dilutions of gG-1 expressed in Sf9 cells by vectors Ac373'gG-1 and AcDSMgG-1. The dilution factor is shown to the right. Cell extracts similar to those used in Panel A were four-fold serially diluted in phosphate-buffered saline, bound to a nitrocellulose membrane using a

slot-blot apparatus, and reacted with the gG-1-specific monoclonal antibody used in Panel A (H1379). As can be clearly seen in the slot-blot, immunoreactive protein diluted by a factor of 64 was detected in the cell extracts produced by the novel baculovirus vector AcDSMgG-1, whereas immunoreactive protein produced by the Ac373'gG-1 vector was only detectable up to a dilution factor of 16. Therefore, the level of expression of gG-1 by the baculovirus vector AcDSMgG-1 was approximately four-fold greater than the level of gG-1 expression by the Ac373'gG-1 vector. It is well known by those skilled in the art that a recombinant protein produced at a higher level of expression provides a purer product. Thus, gG-1 and gG-2 antigens produced by the novel baculovirus vector recited in the product-by-process limitation can be isolated in a much purer form than gG-1 and gG-2 antigens produced by other putative baculovirus vectors.

Purity is highly relevant to and especially important for the utility of antigens. The ability to use an antigen in a diagnostic assay depends upon the purity of the antigen. If an antigen lacks purity, it may be useless in diagnostic assays because of false positive signals caused by the binding of antibodies to impurities. HSV gG-1 and gG-2 antigens are especially useful in diagnostic assays because they are unique to type-1 and type-2 HSV, respectively. Therefore, an increase in the purity of the recombinant antigens produced by the process recited in the claims of Group II increases their utility. This increased purity over HSV gG-1 and gG-2 antigens produced by other putative baculovirus vectors makes the claimed antigens produced by the recited baculovirus vector patentably distinct from the antigens produced by other putative baculovirus vectors.

2. The gG-1 and gG-2 antigens defined by the product-by-process limitation are structurally different than gG-1 and gG-2 antigens produced by other putative baculovirus vectors.

It was unexpectedly discovered that the novel baculovirus vector AcDSMgG-1 not only produces more glycoprotein than the Ac373'gG-1 vector, indicating

a higher level of expression, but the novel baculovirus vector produces proteins having different electrophoretic band patterns than produced from the Ac373'gG-1 vector, indicating that the recombinant proteins are structurally different. These observations are explained on page 233, left column, first full paragraph, of the *Virology* paper, which is reproduced as follows:

> Expression of the recombinant gG-1's differed in two respects. (i) The intensity of the reaction with both antibodies was higher in extracts of cells infected with AcDSMgG-1 than with Ac373'gG-1 (Fig. 2A). (ii) In extracts of Sf9 cells infected with AcDSMgG-1, 42K reacted more than 37K. In extracts of Sf9 cells infected with Ac373'gG-1, the opposite was true, with 42K being very faint.

These results are best shown in Figure 2 of the *Virology* paper (page 233). Panel A of Figure 2 shows photographs of two electrophoretic gels. The gel on the left was reacted with antibodies from human serum identified as HSV-1 positive and HSV-2 negative. The gel on the right was reacted with a monoclonal antibody specific for gG-1 (H1379). The left two lanes of each gel represent SDS-PAGE separations of the glycoprotein products of vector Ac373'gG-1 and novel vector AcDSMgG-1, respectively, with molecular mass standards shown on the left side of each gel. The remaining two lanes of each gel are controls.

In both the human serum reactive and the monoclonal antibody reactive gels, the glycoprotein product of vector Ac373'gG-1 shows a high intensity band at approximately 37 kDa and a low intensity band at 42 kDa. In contrast, the glycoprotein product produced by the novel vector AcDSMgG-1 shows a very high intensity band at approximately 42 kDa and a lower intensity band at approximately 37 kDa. It is interesting to note that the 37 kDa bands for both the Ac373'gG-1 and AcDSMgG-1 have similar intensities, demonstrating that the differences in intensities for the 42 kDa band are due to

physical differences in the proteins rather than the overall increase in expression by the AcDSMgG-1 vector.

In conclusion, the data described above clearly demonstrate that the glycoprotein gG-1 produced from the novel baculovirus vector AcDSMgG-1 is physically different from the gG-1 glycoprotein produced by the baculovirus vector Ac373'gG-1. In addition, the level of expression of gG-1 by the novel baculovirus vector AcDSMgG-1 is significantly higher, thereby resulting in a purer product.

Current law, as reflected by MPEP § 2113, allows patent applicants to claim their invention using product-by-process claims. One of the reasons product-by-process are acceptable is to allow inventors to claim their inventions even when the exact structure of the product cannot be shown. Patent appplicants in these cases merely have to show that their product is patentably distinct over the prior art products. By refusing to accept the proffered evidence of patentable distinctions and by continuing to require that Appellants submit exact structures of the products of the invention, the Examiner is essentially refusing to allow Appellants to claim their invention by way of product-by-process claims.

B. The novel baculovirus vector recited in the product-by-process limitation present in the claims of Group II is unobvious over the cited references.

The Examiner argued that it would have been obvious to one skilled in the art at the time the invention was made to construct a baculovirus vector meeting the requirements recited in the product-by-process limitation present in the claims of Group II. However, the cited references do not make the novel baculovirus obvious. A combination of the cited references with the knowledge and ability of one of ordinary skill would not have been enough to produce the novel baculovirus vector. The teachings of the references combined with the knowledge of one of ordinary skill in the art at the time the invention was made would have been insufficient.

None of the cited references, alone or in combination, teach how to construct the baculovirus vector recited in the product-by-process limitation present in the claims of Group II. The mere suggestion by Matsuura et al. of the problem to be solved, namely constructing a plasmid that faithfully reproduces a 5' nontranslated polyhedrin leader sequence, is nothing more than a wish or a desire for a result. Such a desire or wish is legally insufficient without a disclosure of the means to carry out the invention. See Hybritech, Inc. v. Monoclonal Antibodies, Inc., 801 F.2d 1367, 231 U.S.P.Q. 81 (Fed. Cir. 1986) (Invitations to try to accomplish something do not show obviousness since they "do not suggest how that end might be accomplished.").

Matsuura et al. do not teach or suggest a way to accomplish the solution provided by the present specification. In fact, Matsuura et al. disclose 12 plasmids (including pAc373 of Smith et al.) with different 5' nontranslated leader sequences, but none that succeeded in precisely replicating the 5' nontranslated sequence and translation initiation codon of the wild-type baculovirus. Thus, Matsuura et al., after disclosing an incentive to obtain the presently recited baculovirus vector and numerous examples of failed attempts, could not provide a way to make pAcDSM, because, until the disclosure of the present specification, it was not obvious how to accomplish that goal. Moreover, the very reference which the Examiner alleges to provide motivation could not overcome the scientific hurdles to produce that which was desired and which appellants have provided.

The case law is clear that an Examiner cannot use hindsight based on the applicant's own teaching of the invention to fill in the gaps in the prior art. See In re Gorman, 933 F.2d 982, 18 U.S.P.Q.2d 1885, 1888 (Fed. Cir. 1991) ("[I]t is impermissible ... simply to engage in a hindsight reconstruction of the claimed invention, using the applicants' structure as template in selecting elements from references to fill in the gaps.").

The case law is very clear that a teaching to form a basis of an obvious rejection must be in the cited references. See In re Dow Chemical Co., 837 F.2d 469, 5

U.S.P.Q. 2d 1529, 1531 (Fed. Cir. 1988) ("The consistent criterion for determination of obviousness is whether the prior art would have suggested to one of ordinary skill in the art that this process should be carried out and would have a reasonable likelihood of success, viewed in the light of the prior art ... Both the suggestion and the expectation of success must be found in the prior art, not in the applicant's disclosure."). See also Northern Telecom, Inc. v. DataPoint Corp., 908 F.2d 931, 15 U.S.P.Q.2d. 1321, 1344 (Fed. Cir. 1990) ("Whether the changes from the prior art are 'minor', ... the changes must be evaluated in terms of the whole invention, including whether the prior art provides any teaching or suggestion to one of ordinary skill in the art to make the changes that would produce the patentees' [invention]."). Such a specific teaching is simply not to be found in the cited references.

In addition, another key fact missing from the Examiner's argument is that all the tools to carry out the claimed invention are not in the cited art or the prior art generally. Appellants had to synthesize and utilize reagents not available to the skilled artisan in order to make the plasmid necessary for the construction of the presently recited baculovirus vector. More specifically, the specific plasmid pAcDSM can be used as a transfer vector to insert a foreign gene precisely at the translation initiation codon of the polyhedrin gene, without either missing any nucleotide present in the initiation codon or introducing any extraneous nucleotide at the initiation codon site. The plasmid pAcDSM, as defined in the specification, contains a specific nucleic acid, the creation of which required the generation of a novel synthetic oligonucleotide (V78) not suggested by the cited references. V78 is a 32 base pair (excluding overhang) double stranded synthetic oligonucleotide devised by the inventors to include 6 restriction sites to be used in the inventors' scheme for designing a plasmid that could receive a foreign gene immediately downstream of the translation initiation codon while maintaining the native 5' nontranslated leader sequence. Not only did V78 have to be devised to accommodate insertion of a foreign gene precisely downstream of a translation initiation codon, but it also had to be

constructed so that it could be inserted into a plasmid containing the polyhedrin promoter without disrupting any other sequences in the 5' nontranslated leader sequence of the promoter. The inventors then used V78 in combination with known restriction enzymes in a series of many steps not suggested in any of the cited references to provide a site in pAcDSM for foreign gene insertion, which resulted in a recombinant baculovirus having the characteristics set forth in the claims of Group II. Thus, where others had failed, Appellants designed a novel oligonucleotide which was an important step in achieving the claimed recombinant antigens.

Additionally, the amount of experimentation required to make the invention should be considered to make a case of obviousness. In re Dow Chemical Co., 5 U.S.P.Q.2d 1529 (Fed. Cir. 1988); Westnofa USA v. Whole Life Co., Inc., 3 U.S.P.Q.2d 1352, 1354, 1355 (D.C. Massachusetts, 1987) ("The significant resources devoted to developing this ... [invention] ... and the fact that development extended over three years support an inference that this invention was not obvious. The substantial efforts made by the inventors also indicate that the invention was not easily made by one skilled in the art."). Also, by inference from the enablement context, it is clear that an invention should not be considered obvious if the amount of experimentation required to obtain it was undue experimentation. Hybritech, Inc. v. Monoclonal Antibodies, Inc., 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986) ; Amgen Inc. v. Chugai Pharmaceutical Co., Ltd., 18 U.S.P.Q.2d 1016, 1023 (Fed. Cir. 1991) (In supporting the lower court's conclusion that the subject invention was unobvious, the court noted the testimony of an expert to the effect that "it would have been 'difficult' to find the gene." The lower court had found that "no one had successfully screened a genomic library using fully degenerate probes of such high redundancy as the probes used by [the inventor]").

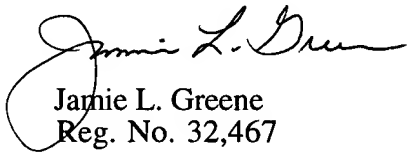
Since the early years of the use of the polyhedrin promoter and especially since the articulation by Matsuura in 1987 of the purported advantages of maintaining the native promoter arrangement, the need for the present invention has clearly been felt in the

art. In the rapidly expanding commercial and scientific fields of recombinant proteins, given the importance of the molecular biological tool provided by the present invention during the many years prior to the present invention, this need was long felt and unsatisfied until the disclosure by Appellants in the present specification.

V. Conclusion

For the reasons argued herein, Appellants request that the Examiner's rejection be reversed and the pending claims be allowed.

Respectfully submitted,



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Appendix

CLAIMS ON APPEAL

7. Pure recombinant herpes simplex virus gG-1 antigen produced by employing a recombinant baculovirus having the 5' nontranslated leader sequence of the polyhedrin gene joined to the coding region of a foreign gene precisely at the translation initiation codon of the polyhedrin gene, without either missing any nucleotide present in said initiation codon or introducing any extraneous nucleotide at the initiation codon site, wherein said foreign gene is herpes simplex virus type 1 glycoprotein gene.

8. Pure recombinant herpes simplex virus gG-2 antigen produced by employing a recombinant baculovirus having the 5' nontranslated leader sequence of the polyhedrin gene joined to the coding region of a foreign gene precisely at the translation initiation codon of the polyhedrin gene, without either missing any nucleotide present in said initiation codon or introducing any extraneous nucleotide at the initiation codon site, wherein said foreign gene is herpes simplex virus type 2 glycoprotein gene.

16. A composition comprising pure recombinant baculovirus expressed herpes simplex virus gG-1 antigen or herpes simplex virus gG-2 antigen in a pharmaceutically acceptable carrier.

17. The composition of Claim 16, wherein the recombinant baculovirus has the 5' nontranslated leader sequence of the polyhedrin gene joined to the coding region of a herpes simplex virus type 1 or type 2 glycoprotein gene precisely at the translation initiation codon of the polyhedrin gene, without either missing any nucleotide present in the initiation codon or introducing any extraneous nucleotide at the initiation codon site.

18. The composition of Claim 16, wherein the recombinant herpes simplex virus gG-1 antigen has a molecular weight selected from the group consisting of approximately 42 kDa, 43 kDa, and combinations thereof.

19. The composition of Claim 16, wherein the recombinant herpes simplex virus gG-2 antigen has a molecular weight selected from the group consisting of approximately 107 kDa, 118 kDa, 128 kDa, 143 kDa, and combinations thereof.

20. The composition of Claim 7, wherein the recombinant herpes simplex virus gG-1 antigen has a molecular weight selected from the group consisting of approximately 42 kDa, 43 kDa, and combinations thereof.

21. The composition of Claim 8, wherein the recombinant herpes simplex virus gG-2 antigen has a molecular weight selected from the group consisting of approximately 107 kDa, 118 kDa, 128 kDa, 143 kDa, and combinations thereof.

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Expression of HSV-1 and HSV-2 Glycoprotein G in Insect Cells by Using a Novel Baculovirus Expression Vector

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Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) glycoprotein G (gG-1 and gG-2) were expressed in insect cells from recombinant baculoviruses (AcDSMgG-1 and AcDSMgG-2, respectively) constructed using a novel baculovirus transfer vector, pAcDSM. This vector allows the coding region of a foreign gene to be precisely linked to the baculovirus polyhedrin gene at the translation initiation site and retains the native polyhedrin translation initiation environment. Fourfold more gG-1, with a higher ratio of glycosylated to unglycosylated product, was produced by AcDSMgG-1 than by Ac373'gG-1, a recombinant baculovirus which differs from AcDSMgG-1 by the presence of 21 extraneous nucleotides in the 5' nontranslated sequence. gG-1 and gG-2 expressed in recombinant baculovirus-infected insect cells undergo cotranslational N-linked glycosylation, but the overall processing of the proteins differs from that observed in HSV-1- or HSV-2-infected cells. Despite these differences, baculovirus-expressed gG-1 and gG-2 were recognized in a HSV type-specific manner by human serum specimens.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) are genetically very similar (Kieff *et al.*, 1972; Ludwig *et al.*, 1972), resulting in extensive antigenic cross-reactivity (reviewed by Nahmias and Dowdle, 1968; and Honess and Watson, 1977). This cross-reactivity has resulted in many serologic tests for distinguishing prior infection with HSV-1 from that with HSV-2 being laborious as well as frequently inconclusive, particularly with specimens containing antibodies to both viruses (Nahmias *et al.*, 1970).

The identification of glycoprotein G as a type-specific antigen (gG-1) (Ackermann *et al.*, 1986; Richman *et al.*, 1986) (and gG-2) (Roizman *et al.*, 1984; Marsden *et al.*, 1984) allowed the development of accurate type-specific serologic assays (Lee *et al.*, 1985, 1986; Nahmias *et al.*, 1986; Ashley *et al.*, 1988). Studies using these assays have clearly demonstrated the accuracy that can be obtained using reactivity with gG-1 and gG-2 as the basis for the assay, but because of difficulties in preparing and standardizing reagents, the tests have been performed routinely in few laboratories. To obtain abundant supplies of well-characterized antigen for use in serologic tests and in studies of the host immune response to gG, and to bypass large-scale culturing of pathogenic agents, we have expressed gG-1 and gG-2 in the baculovirus expression system (reviewed by Luckow and Summers, 1988).

Although high levels of gene expression have been obtained using the baculovirus system, in only a few cases has the level of gene expression approached that of native polyhedrin. Kozak (1981) has shown that sequences immediately surrounding the translation initiation site can have a profound effect on the efficiency of translation initiation. We hypothesized that at least some of the differences in the level of gene expression between native polyhedrin and foreign genes inserted in its place may be due to missing or extraneous nucleotides in the vicinity of the translation initiation codon in vectors currently in use (Smith *et al.*, 1985; Matsuura *et al.*, 1987; Luckow and Summers, 1989). Thus the most efficient expression of a foreign gene in this system might occur if the native polyhedrin sequences controlling regulation of transcription and translation embedded in the 5' nontranslated leader sequence were unaltered and joined to the coding region of the foreign gene precisely at the translation initiation site, with no missing or extraneous nucleotides.

We describe in this report (i) the creation of a baculovirus transfer vector that fulfills the above requirements, (ii) its application for the construction of recombinant baculoviruses expressing gG-1 and gG-2 in insect cells, and (iii) the characterization and type specificity for HSV antibodies in human serum specimens of baculovirus-expressed gG-1 and gG-2.

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MATERIALS AND METHODS

Cells and viruses

Wild-type and recombinant AcNPV were grown and assayed in a continuous ovarian cell line (Sf9) derived from *Spodoptera frugiperda* (fall armyworm), as previously described (Summers and Smith, 1987). Sf9 cells (ATCC No. CRL 1711) were obtained from the American Type Culture Collection (Rockville, MD). Wild-type AcNPV and the gene transfer vector pAc373 (Smith *et al.*, 1985) were obtained from Dr. Max Summers, Texas A&M University (College Station, TX). HSV-1(F) and HSV-2(G) (Ejercito *et al.*, 1968) were obtained from Dr. Bernard Roizman, University of Chicago (Chicago, IL) and grown and propagated as described (Morse *et al.*, 1977). Monoclonal antibodies specific for gG-1 (H1379) (Lee *et al.*, 1986) and specific for gG-2 (H1206) (Lee *et al.*, 1985) were obtained from Dr. Lenore Pereira, University of California, San Francisco (San Francisco, CA).

DNA manipulations

DNA manipulations were carried out essentially as described (Maniatis *et al.*, 1982). Restriction endonucleases and T4 DNA polymerase were purchased from New England BioLabs (Beverly, MA), mung bean exonuclease from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ), and T4 DNA ligase from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Oligonucleotides were synthesized in a Model 380 DNA synthesizer from Applied Biosystems (Foster City, CA).

Construction of modified gene transfer vectors

pPP-2. pPP-1 was constructed by digesting pUC8 (Vieira and Messing, 1982) with *Nar*I and filling in the overhanging 5′ nucleotides using T4 DNA polymerase, followed by self-ligation, resulting in the elimination of the *Nar*I site. A synthetic oligoduplex AB (Fig. 1A) was ligated between the *Eco*RI and the *Hin*dIII sites of pPP-1, to obtain pPP-2. This vector can be used to construct gene fusions at any of the three reading frames by using blunt-end ligations at the appropriate site. The fused gene would then be removed from the vector by digestion at the flanking *Eco*RI and *Hin*dIII sites and inserted into a baculovirus transfer vector such as pAc373 at the desired site, using blunt ends if necessary.

pAcDSM. pAc373 was digested to completion with *Sal*I and *Kpn*I. The DNA fragment spanning the region between 3.18 and 4.43 kilobase pairs (kbp) in the coordinate system of Summers and Smith (1987) was purified from agarose and inserted into pUC19 (Norrander *et al.*, 1983) that had previously been digested with the same enzymes. The resulting plasmid (pDM1) was linearized by digestion with *Ava*I. After PEG precipitation (Sadhu and Gedamu, 1988), 5′ overhanging nucleotides were removed by digestion with 150 units of mung bean exonuclease/μg of DNA to obtain blunt ends, followed by *Kpn*I digestion. A synthetic oligoduplex, V78 (Fig. 1A), was inserted between the *Kpn*I site and the blunt end (nucleotide −9 of the 5′ leader sequence of the polyhedrin gene) of pDM1 to obtain pDM2. The fidelity of the construct was checked by nucleotide sequencing using primers flanking the cloned fragment. pDM2 was digested with *Eco*RV and *Kpn*I. The resulting 118-bp fragment was ligated to pAc373 previously digested with the same enzymes to obtain the transfer vector pAcDSM.

In order to use pAcDSM, it is digested with *Pst*I, treated with T4 DNA polymerase to trim the 3′ overhanging nucleotides to a blunt-ended C at position −9 of the polyhedrin 5′ nontranslated leader sequence, and then digested at another restriction site in the polylinker using an enzyme which generates a cohesive terminus, allowing for efficient directional insertion of a foreign gene (Fig. 1B). The foreign gene is modified for compatibility with the vector by assembling a segment of DNA containing, from 5′ to 3′, the nucleotides −8 to −1 of the 5′ nontranslated leader sequence of the polyhedrin gene, the translation initiation codon, the coding region of the foreign gene, the downstream region through the polyadenylation signal, and a unique restriction site compatible with one in the transfer vector's polylinker. Segments of DNA containing these features can be constructed by using standard cloning techniques and synthetic oligonucleotides as was done here, by total synthesis using long synthetic oligonucleotides, or by using the polymerase chain reaction (Saiki *et al.*, 1985) with appropriately tailed primers. When the modified segment of DNA is inserted into pAcDSM prepared as described above, the C in position −9 of the polyhedrin 5′ nontranslated leader sequence is linked to the first nucleotide of the modified gene (C in position −8), regenerating the 5′ leader sequence of the polyhedrin gene.

Cloning gG-1 and gG-2 into baculovirus transfer vectors

For convenience, the nucleotide sequence coordinates reported for the genomic sequence of HSV-1 strain 17 (McGeoch *et al.*, 1988) and for the *Hin*dIII L fragment of HSV-2 strain HG52 (McGeoch *et al.*, 1987) are used throughout this paper. HSV-1 strain F (HSV-1(F)) and HSV-2 strain G (HSV-2(G)) DNAs were separately digested to completion with *Bam*HI and shotgun cloned into pUC19. Plasmids carrying HSV-1(F) *Bam*HI

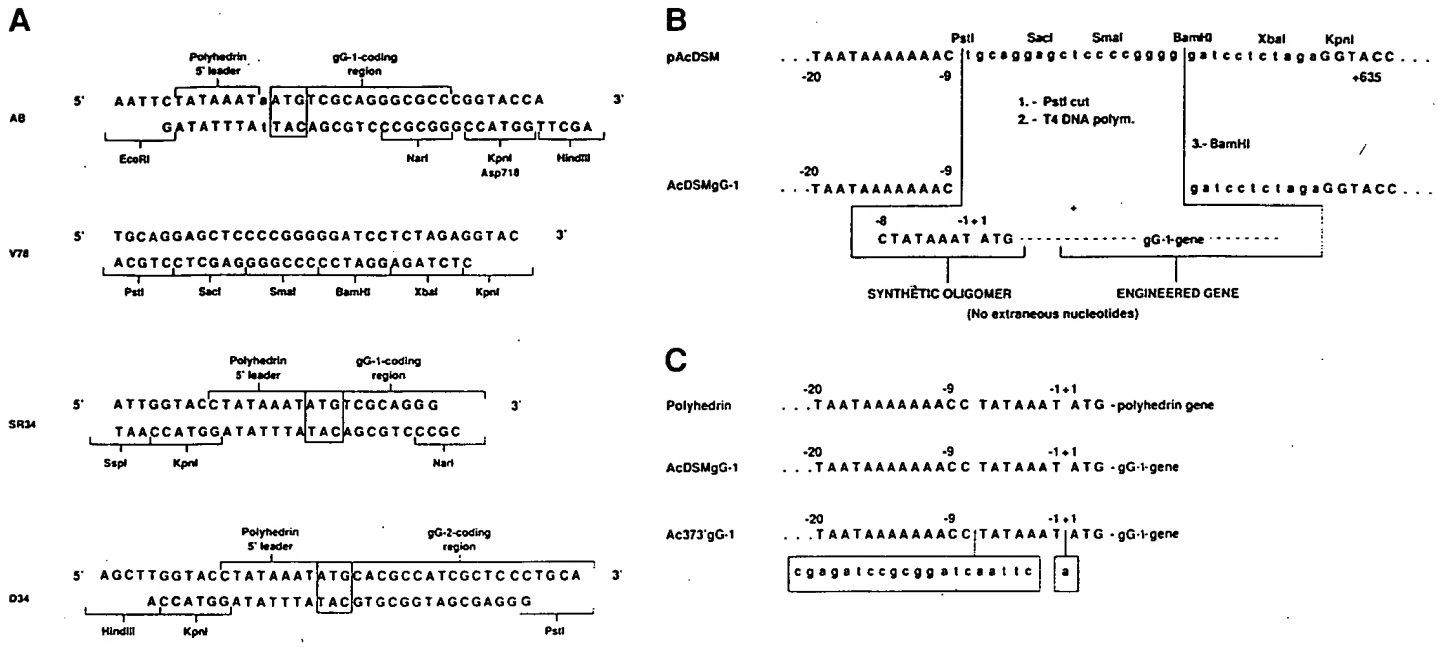

FIG. 1. (A) Synthetic oligomers used in the construction of the baculovirus gene transfer vectors and the gG-1- and gG-2-expressing recombinant baculoviruses. Oligoduplex AB was used in the construction of pPP-2, oligoduplex V78 for pAcDSM, oligoduplex SR34 for AcDSMgG-1, and oligoduplex D34 for AcDSMgG-2. Relevant restriction endonuclease sites are indicated. (B) Schematic representation of the method of inserting a foreign gene into the transfer vector pAcDSM. (C) Comparison of nucleotide sequences in the 5' nontranslated region of the wild-type baculovirus (AcNPV) and the recombinant viruses Ac373'gG-1 and AcDSMgG-1. Extraneous nucleotides relative to the wild-type polyhedrin sequence are boxed. The a preceding the initiation codon in Ac373'gG-1 was incorporated into the construct based on a published sequence (Hooft Van Iddekinge *et al.*, 1983) later found to be in error (Howard *et al.*, 1986).

J (pH1F-110, nucleotides 136,285 to 142,742) and HSV-2(G) *Bam*HI L (pH2G-112, nucleotides 2356 to 6894) fragments, which contain the intact gG-1 and gG-2 genes, respectively, were used as the starting point for engineering the genes for compatibility with the transfer vectors.

gG-1 insertion into pPP-2. pH1F-110 was digested with *Sph*I to remove nucleotides 137,617 to 142,742 of the HSV-1 fragment (removing two of the three *Nar*I sites in the insert) and ligated to itself. The resulting plasmid (pH1F-1001) was digested with *Nar*I and religated to itself, deleting nucleotides 136,285 to 136,749 and a small portion of the vector, resulting in pH1F-1002. This plasmid was digested with *Nar*I and *Hin*dIII, and the 873-bp fragment carrying the nearly complete gG-1 gene (nucleotides 136,749 to 137,622) was ligated to plasmid pPP-2, which had previously been digested with *Nar*I and *Hin*dIII. The resulting plasmid (pH1F-1011) was digested with *Eco*RI and *Hin*dIII. The fragment containing the modified gG-1 gene was purified from an agarose gel, made blunt-ended with T4 DNA polymerase, and ligated to pAc373, which had been digested with *Bam*HI and made blunt-ended by treatment with T4 DNA polymerase. A plasmid containing the gG-1 gene inserted in the proper orientation was designated pAc373'gG-1.

gG-1 insertion into pAcDSM. The 871-bp fragment between the *Nar*I and the *Sph*I sites of pH1F-1002 (nucleotides 136,749 to 137,620), carrying the nearly complete gG-1 gene, was ligated to pUC18 that had previously been digested with the same enzymes. The resulting plasmid, pSR1, was digested with *Nde*I and *Bam*HI, producing a 944-bp fragment that was ligated to pUC9 that had been previously digested with the same enzymes, resulting in pSR3. A synthetic oligoduplex, SR34 (Fig. 1A), was ligated between the *Ssp*I and the *Nar*I sites of pSR3. The resulting construct, plasmid pSR5, was successively incubated with *Kpn*I, T4 DNA polymerase, and *Bam*HI and ligated with the vector pAcDSM that had previously been successively reacted with *Pst*I, T4 DNA polymerase, and *Bam*HI. The resulting plasmid was designated pAcDSMgG-1.

gG-2 insertion in pAcDSM. pH2G-112 was digested with *Bam*HI and *Pvu*II to obtain a 3779-bp fragment (nucleotides 2356 to 6135) that was purified from agarose and ligated with pUC19 that had been digested with *Bam*HI and *Ssp*I. The resulting plasmid, pDS1, was digested with *Pst*I and *Hin*dIII and ligated with a synthetic oligodupex, D34 (Fig. 1A), to generate pDS2. The 1316-bp fragment (nucleotides 2515 to 3831) resulting from digesting pH2G-112 with *Hin*cII was purified from agarose and successively reacted with *Bst*NI,

T4 DNA polymerase, and *Sty*I. The resulting 161-bp fragment (nucleotides 2859 to 3020) was purified from agarose and then ligated to pDS2, which had previously been successively incubated with *Pst*I, T4 DNA polymerase, and *Sty*I. The resulting plasmid, pDS6, was digested with *Ssp*I and *Xho*I and ligated to an agarose-purified 2148-bp fragment obtained by digesting pH2G-112 with *Ssp*I and *Xho*I (nucleotides 2983 to 5131). The resulting plasmid, pDS7, was digested with *Hin*dIII and *Xho*I. The resulting 2311-bp fragment (carrying the complete gG-2 gene plus flanking plasmid-derived sequences) was ligated with pUC9 that had been digested with *Hin*dIII and *Sal*I. The resulting plasmid, pDS8, after successive reactions with *Kpn*I, T4 DNA polymerase, and *Bam*HI, was ligated with pAcDSM that had been successively reacted with *Pst*I, T4 DNA polymerase, and *Bam*HI to obtain pAcDSMgG-2.

Transfection and selection of recombinant baculoviruses

Procedures for transfection, selection of recombinant baculoviruses, and virus titration were performed as described (Summers and Smith, 1987). Recombinant viruses were plaque purified at least five times. Proper insertion of the transferred genes into the baculovirus genome was confirmed by blot hybridization analysis of the recombinant virus genomes (data not shown).

Protein analysis by immunoblots

Sf9 cells were grown either in Hink's medium supplemented with 10% fetal calf serum according to the method of Summers and Smith (1987) or in serum-free medium (Excell 400, J. R. Scientific, Woodland, CA) and infected with recombinant or wild-type baculoviruses at a multiplicity of infection of 10 PFU/cell in 25-cm^2 flasks at 27°. After 1.5 hr, the inoculum was removed and replaced with fresh medium. At the appropriate times postinfection, the cells were scraped from the flask and harvested by centrifugation for 10 min at 1500 *g* at 4°. The pellet was resuspended in disruption buffer (5.7 *M* urea, 2.8% SDS, and 1.8 *M* 2-mercaptoethanol), sonicated for 30 sec (output control 4, duty cycle 50%) in a cup horn sonicator (Model W-375, Heat Systems-Ultrasonic, Inc., Farmingdale, NY), and heated for 3 min at 95°. Proteins were separated by electrophoresis in polyacrylamide gels (Laemmli, 1970) (acrylamide to bis-acrylamide ratio of 37.5:1) and then electrically transferred onto nitrocellulose paper (BA85, Schleicher and Schuell) (Towbin *et al.*, 1979) using 0.1% SDS in the transfer buffer. Blots were incubated for 1 hr in blotto (5% skim milk, 0.01 *M* phosphate-buffered saline, pH 7.4, and 0.05% Tween 20) and then incubated for 1 hr in fresh blotto containing the appropriate dilution of either human serum or monoclonal antibody. After three 10-min washes in 0.05% Tween 20 in phosphate-buffered saline, blots were incubated with alkaline phosphatase-conjugated goat anti-human or alkaline phosphatase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Rockville Centre, NY) in 0.05% Tween 20 in phosphate-buffered saline for 2 hr, washed three times for 10 min with the same buffer, and developed with *p*-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (Bio-Rad) according to the vendor's protocol.

RESULTS

Vector construction

Two gene transfer vectors were constructed, pPP-2, which incorporates nucleotides −7 to −1 of the 5′ nontranslated leader sequence of the polyhedrin gene missing in the widely used transfer vector pAc373 (Smith *et al.*, 1985) but which results in recombinant baculoviruses containing 21 extraneous nucleotides in this region, and pAcDSM, which allows the construction of recombinant baculoviruses with the 5′ nontranslated leader sequence of the polyhedrin gene joined precisely to the coding region of the foreign gene at the translation initiation codon, with no missing or extraneous nucleotides. pPP-2 was constructed early in the course of these studies. It is awkward to use and offers few advantages relative to other currently available vectors. Its description is included here because it was used to construct the recombinant baculovirus Ac373′gG-1.

Construction of recombinant baculoviruses expressing gG-1 and gG-2

As described under Materials and Methods, recombinant baculoviruses Ac373′gG-1 and AcDSMgG-1 expressing gG-1 were constructed using pPP-2 in conjunction with pAc373 and pAcDSM, respectively. The nucleotide sequence in the vicinity of the translation initiation codon of these viruses is shown in Fig. 1C. A recombinant baculovirus expressing gG-2, AcDSMgG-2, was created using pAcDSM.

Synthesis and processing of baculovirus-expressed gG-1

Replica immunoblots of extracts from recombinant-infected, wild-type-infected, or uninfected Sf9 cells were reacted with either a human serum specimen that had been identified as HSV-1-positive and

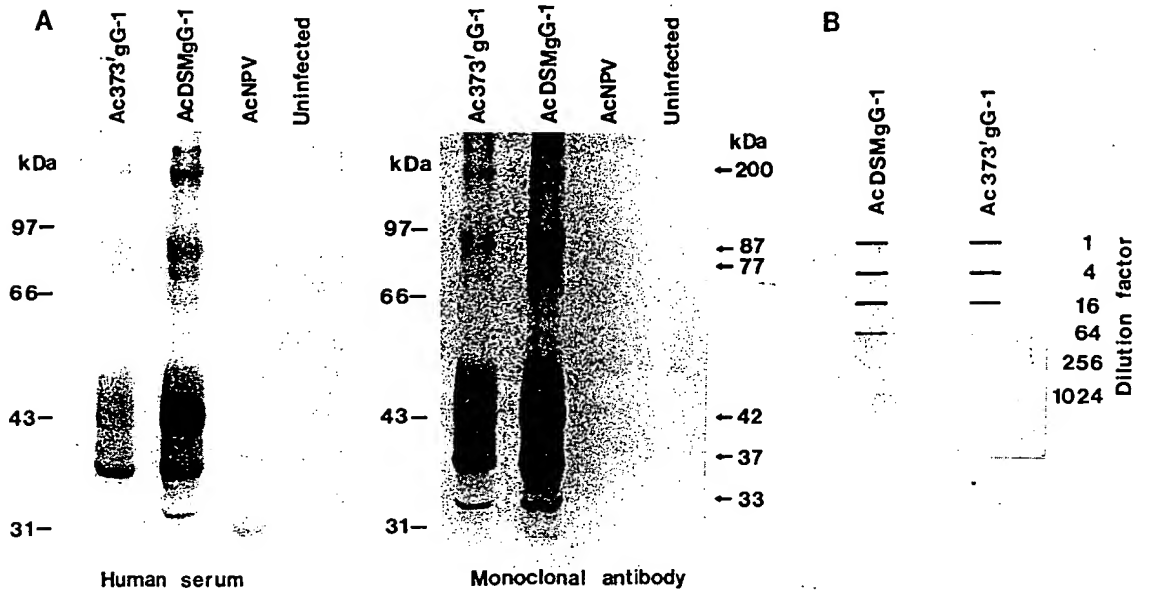

FIG. 2. Reaction of baculovirus-expressed gG-1s with antibodies. (A) Proteins extracted at 100 hr p.i. from equal numbers of Ac373'gG-1-, AcDSMgG-1-, and AcNPV-infected or uninfected Sf9 cells were separated by SDS–PAGE in 11% gels, transferred to nitrocellulose membranes, and then tested with the indicated antibodies. The positions of the molecular mass standards are shown on the side of each panel, (phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 42.7 kDa; and carbonic anhydrase, 31 kDa). The apparent molecular mass of gG-1-related species (arrows) is indicated. (B) Quantitative comparison of the amount of gG-1 expressed in Sf9 cells by the recombinants Ac373'gG-1 and AcDSMgG-1. Cell extracts similar to those used in A were fourfold serially diluted in 0.01 *M* phosphate-buffered saline, pH 7.4, bound to a nitrocellulose membrane using a slot-blot apparatus, and reacted with gG-1-specific monoclonal antibody (H1379).

HSV-2-negative using an HSV type-specific indirect hemagglutination assay (IHA) (Bernstein and Stewart, 1971) or a monoclonal antibody specific for gG-1 (H1379) (Lee *et al.*, 1986) (Fig. 2A). None of the antibodies reacted with proteins in the lanes containing proteins from AcNPV-infected or uninfected cells, except for a weak reaction between some human serum specimens and polyhedrin in AcNPV-infected cells. The pattern of reactivity with both antibodies was identical in the lanes containing the gG-1 recombinant-infected cell extracts (lanes Ac373'gG-1 and AcDSMgG-1). The major reacting bands appeared at 37 and 42 kDa apparent molecular mass (37K and 42K) within a region of diffuse reactivity between 36 and 48 kDa apparent molecular mass.

Expression of the recombinant gG-1s differed in two respects. (i) The intensity of the reaction with both antibodies was higher in extracts of cells infected with AcDSMgG-1 than with Ac373'gG-1 (Fig. 2A). (ii) In extracts of Sf9 cells infected with AcDSMgG-1, 42K reacted more than 37K. In extracts of Sf9 cells infected with Ac373'gG-1, the opposite was true, with 42K being very faint.

Slot-blot analysis was used to eliminate difficulties inherent in quantifying multiple diffuse bands in electropherograms. The intensity of the reaction was about fourfold greater in extracts harvested from cells infected with AcDSMgG-1 than with Ac373'gG-1 at either 100 hr p.i. (Fig. 2B) or 72 hr p.i. (data not shown). Thus more gG-1 was expressed from the construct that mimicked the polyhedrin 5' nontranslated leader sequence (AcDSMgG-1) than from the construct that contained 21 extraneous nucleotides in this region (Ac373'gG-1).

We monitored the levels of gG-1 production and the ratios of intensity between the two major gG-1 bands in the two recombinants as a function of time after infection (Fig. 3A). The major gG-1 bands (37K and 42K) were first detected at 36 hr p.i. At all time points, more gG-1 was detected in the lanes containing extracts from cells infected with AcDSMgG-1 (Fig. 3A, lanes b). The maximum expression with both recombinants occurred at about 72 hr p.i. As was previously seen at 100 hr p.i., at all time points 42K was more abundant than 37K in AcDSMgG-1-infected cells (lanes b), while in Ac373'gG-1-infected cells (lanes a), 37K was more abundant than 42K. This indicates that the difference in processing of gG-1 may be due to differences between the transfer vectors. Similar patterns and levels of synthesis were observed in two independently plaque-purified progeny of the transfection that produced Ac373'gG-1 (data not shown).

Treatment of AcDSMgG-1-infected Sf9 cells with tunicamycin, an inhibitor of a precursor necessary for

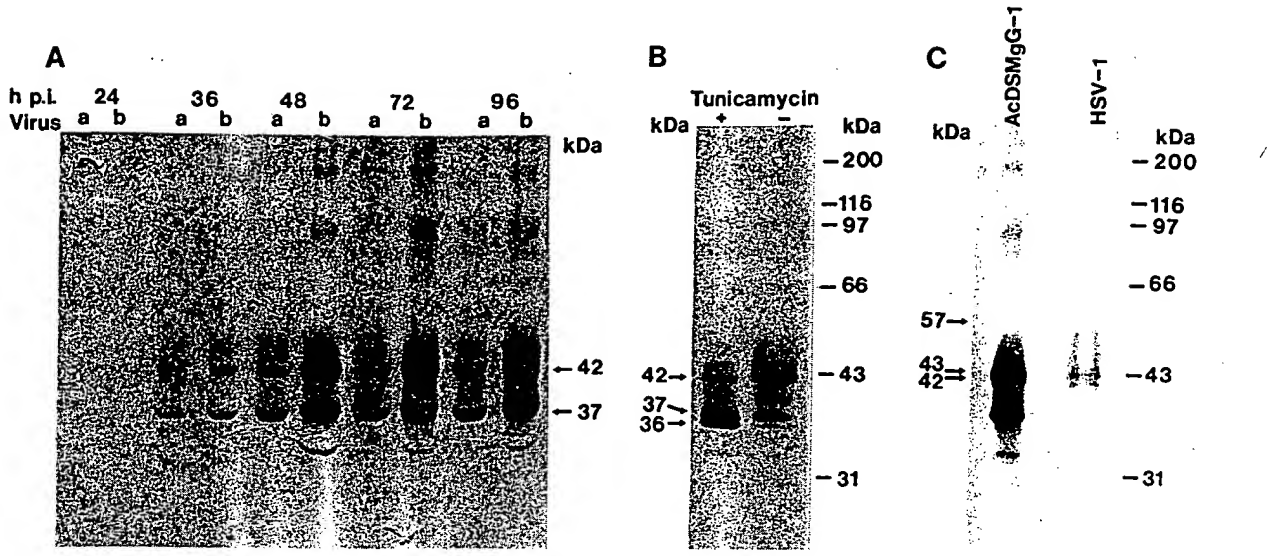

FIG. 3. Synthesis and processing of baculovirus-expressed gG-1. (A) Time course of the synthesis of gG-1 in Ac373'gG-1-infected and AcDSMgG-1-infected (lanes a and b, respectively) Sf9 cells. Cells were harvested at the indicated times, treated as those in Fig. 2A, and reacted with a HSV-1-positive human serum specimen. (B) Immunoblot of proteins extracted from Sf9 cells infected with AcDSMgG-1 and grown in the presence (+) or the absence (−) of 3 μg/ml tunicamycin from 24 hr p.i. until 54 hr p.i. Blots were reacted with gG-1-specific monoclonal antibody (H1379). (C) Immunoblot analysis of proteins extracted from Sf9 cells infected with AcDSMgG-1 and from HEp-2 cells infected with HSV-1(F); about 100-fold more infected cells of the latter were used. Myosin (200 kDa) and β-galactosidase (116.3 kDa) were used in addition to the molecular mass standards used in Fig. 2. The apparent molecular mass of bands discussed in the text (arrows) is indicated.

N-linked glycosylation (Hemming, 1982), resulted in a great reduction in the intensity of all the bands over 37 kDa apparent molecular mass, most prominently 42K, and an increase in the intensity of an otherwise very faint species of 36 kDa apparent molecular mass (36K) (Fig. 3B). The abundant 37K is of similar intensity in infected cells cultured either in the presence or in the absence of tunicamycin. In heavily loaded gels, species of 77 and 87 kDa apparent molecular mass (77K and 87K) were replaced by species of 74 and 83 kDa (74K and 83K) apparent molecular mass (data not shown).

In a comparison of baculovirus- and HSV-1-expressed gG-1, major bands with apparent molecular masses of 42 and 43 kDa (42K and 43K) and a smear between 50 and 57 kDa apparent molecular mass were found in extracts of HEp-2 cells infected with HSV-1(F) (Fig. 3C). In heavily loaded gels, the smear extended to 66 kDa apparent molecular mass (not shown).

Synthesis and processing of baculovirus-expressed gG-2

Replica immunoblots of extracts of Sf9 cells infected with AcDSMgG-2, wild-type baculovirus, or uninfected were reacted with either a HSV-2-positive and a HSV-1-negative human serum specimen or with a gG-2-specific monoclonal antibody (H1206) (Lee *et al.*, 1985) (Fig. 4A). In lanes containing extracts from AcDSMgG-2-infected cells, both antibodies reacted with bands with apparent molecular masses of 107, 118, 128, and 143 kDa (107K, 118K, 128K, and 143K). The human serum specimen reacted most strongly with 128K, and the monoclonal antibody with 118K. This difference in reactivity between the human serum and the monoclonal antibody is not a general phenomenon, inasmuch as other human serum specimens reacted most strongly with 118K (data not shown). In overloaded gels a weakly reactive protein with an apparent molecular mass of 34 kDa (34K) was detected.

We monitored expression of gG-2 in recombinant baculovirus-infected cells as a function of time after infection (Fig. 4B). Only one band (118K, open triangle) was detected at the earliest time point examined (24 hr p.i.). By 36 hr p.i. it had increased in intensity and two faint bands appeared (107K and 128K, solid triangles). From 48 hr p.i. onward, the four bands (107K, 118K, 128K, and 143K) previously seen at 100 hr p.i. (Fig. 4A) were visible, with the maximum accumulation of protein at 72 hr p.i. The weakly reactive 34K protein was first detected at 36 hr p.i.

In the electrophoretic pattern of extracts of Sf9 cells infected with AcDSMgG-2 and treated with tunicamycin, bands migrating with apparent molecular masses of 105, 110, and 120 kDa (105K, 110K, and 120K) were

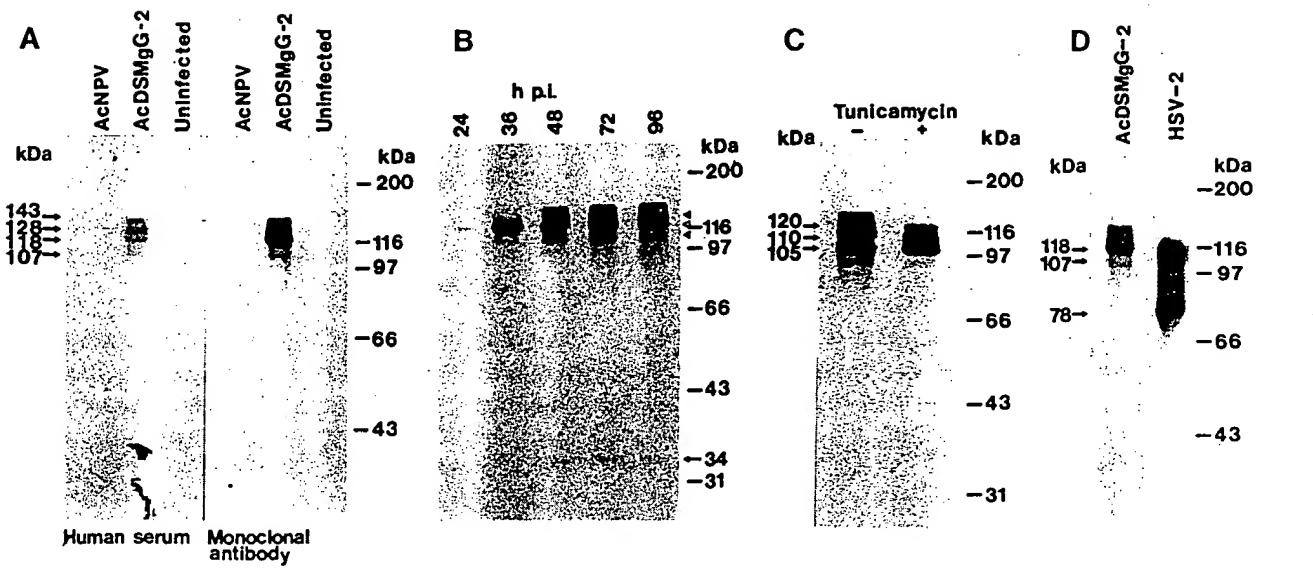

FIG. 4. Synthesis and processing of baculovirus-expressed gG-2. (A) Proteins extracted at 100 hr p.i. were separated in a 9% SDS–PAGE gel, then transferred to nitrocellulose, and reacted with the indicated antibodies. (B) Time course of the synthesis of gG-2 in AcDSMgG-2-infected Sf9 cells. Proteins extracted from cells harvested at the times indicated were treated as those in A and then reacted with gG-2-specific monoclonal antibody (H1206). (C) Immunoblot of proteins extracted from Sf9 cells infected with AcDSMgG-2 and grown in the presence (+) or the absence (–) of 3 μg/ml tunicamycin from 24 to 54 hr p.i. Blots were reacted with gG-2-specific monoclonal antibody (H1206). (D) Immunoblot analysis of proteins extracted from Sf9 cells infected with AcDSMgG-2 and from HEp-2 cells infected with HSV-2(G); about 1.8-fold more infected cells of the latter were used. Molecular mass standards were the same as those for Fig. 3. The apparent molecular mass of bands discussed in the text (arrows or triangles) is indicated.

present, whereas in untreated cells, the 107K, 118K, 128K, and 143K species were seen (Fig. 4C). In overloaded gels, a band migrating with an apparent molecular mass of 30 kDa was present in the extract of infected cells treated with tunicamycin, in contrast with the 34K species seen in untreated cells (data not shown).

In comparisons of baculovirus- and HSV-2-expressed gG-2, in the lane containing extracts from HEp-2 cells infected with HSV-2(G) (Fig. 4D), a smear of reactivity was seen ranging from an apparent molecular mass of 78 through 118 kDa with distinctive species at 78, 107, and 118 kDa apparent molecular mass. In overloaded gels, a faint band with apparent molecular mass of 36 kDa was detected (data not shown).

HSV type specificity of the reaction of human serum specimens with the baculovirus-expressed proteins

Proteins in extracts of Sf9 cells expressing the recombinant gG-1 or gG-2 were tested for reactivity with 10 different human serum specimens previously characterized by using an HSV type-specific IHA (Bernstein and Stewart, 1971). As a representative example, patterns obtained with 3 of these 10 specimens are shown in Fig. 5. Using the gG-1 37K and 42K species and the gG-2 118K species, plus either or both of the 128K and 143K species, as markers of HSV-1 and HSV-2 type-specific reaction, respectively, a serum specimen positive for HSV-1 and negative for HSV-2 by IHA, and a serum specimen positive for HSV-2 and negative for HSV-1 by IHA, each reacted in a type-specific manner in the immunoblot assay (Figs. 5A and 5B, respectively). A serum specimen weakly positive for both types of antibodies by IHA reacted with the gG-1-specific 37K and 42K species (empty triangles) in the lane of the recombinant gG-1, and reacted weakly but clearly with the gG-2 118K and 128K species (full triangles) in the lane containing recombinant gG-2 (Fig. 5C). We have seen various patterns of weak reactivity between human serum specimens and extracts of wild-type baculovirus-infected Sf9 cells as well as uninfected cells. The faint bands seen at 36 kDa apparent molecular mass in the gG-2 lanes of Fig. 5, as well as the bands seen at 170 kDa apparent molecular mass in Fig. 5C, are examples of these reactions. In no case was the reactivity of an extent or nature to lead to ambiguity in interpretation. Results obtained with the other seven serum specimens were in agreement with the IHA results (data not shown).

DISCUSSION

We constructed a transfer vector, pAcDSM, that facilitates the construction of recombinant baculovir-

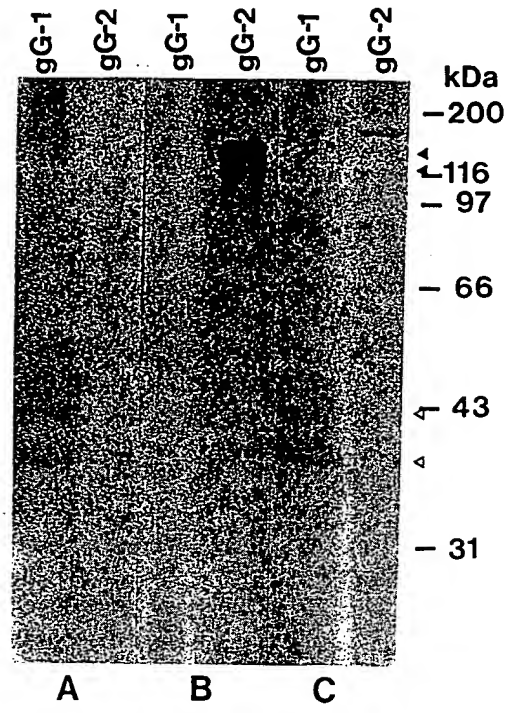

FIG. 5. Immunoblot analysis of the HSV type specificity of the reaction of human serum specimens with AcDSMgG-1- and AcDSMgG-2-infected Sf9 cell extracts. Proteins were separated by SDS–PAGE in a 11% gel, transferred to nitrocellulose, and then reacted with serum specimens known to be HSV-1-positive and HSV-2-negative (A), HSV-1-negative and HSV-2-positive (B), and having a low positive titer to both HSV-1 and HSV-2 (C). Bands considered to be diagnostic for HSV-1-specific reactivity are indicated with open triangles, and those for HSV-2 with solid triangles. Molecular mass standards were the same as those for Fig. 3.

uses containing no missing or extraneous nucleotides in the region 5′ to the translation initiation codon. No other vector reported to date allows such constructs. Although we did not compare the performance of this vector with that of recently described vectors (Matsuura *et al.*, 1987; Luckow and Summers, 1989) which more closely, albeit imperfectly, mimic the gene expression control environment of the native polyhedrin gene than earlier transfer vectors (Smith *et al.*, 1985), we did observe fourfold greater expression of gG-1 from pAcDSM than from a construct that contains 21 extraneous nucleotides in the 5′ nontranslated leader sequence. The nature of pAcDSM will allow careful dissection of the environment in the vicinity of the translation initiation codon through the construction of viruses with precise sequence modifications in this region.

Unexpectedly, in addition to the difference in the level of expression between the two gG-1-expressing recombinants, we observed a difference in the ratio of nonglycosylated precursor (37K, see below) to glycosylated product (42K), with gG-1 expressed from AcDSMgG-1 being the most efficiently processed. Inasmuch as the gG-1 coding sequence was not altered during the construction of the recombinants, these results indicate an effect of mRNA structure on protein processing efficiency. Further studies will be required to test this hypothesis.

Biosynthesis of gG-1

A scenario for the biosynthesis of gG-1 in baculovirus-infected Sf9 cells that accounts for our results is as follows: The 37K species is insensitive to tunicamycin and is likely to be the primary nonglycosylated translation product with its signal peptide uncleaved, indicating that it was not translocated to the lumen of the rough endoplasmic reticulum. The 36K species is most abundant during growth in the presence of tunicamycin and would be the nonglycosylated translation product without its signal peptide. The broad band produced by 42K, which is sensitive to tunicamycin, would be the translation product after having its signal peptide removed and being N-linked glycosylated. Species 77K, 87K, and 200K are likely to be products of further processing. In heavily loaded gels, 77K and 87K were replaced by 74K and 83K species upon treatment with tunicamycin (data not shown), indicating that the generation of the low mobility gG-1-reactive molecules is not dependent on N-linked glycosylation. The low mobility gG-reactive polypeptides are not likely to represent gG oligomers, because cell lysates were boiled in the presence of a detergent, a reducing agent, and urea prior to electrophoresis. The low abundance of the 33K species is likely to be the result of proteolytic degradation.

The biosynthesis of gG-1 in baculovirus-infected insect cells differs in several respects from its synthesis in mammalian cells infected with HSV-1- or gG-1-expressing vaccinia virus recombinants. In a direct comparison of baculovirus- and HSV-1-expressed gG-1, we observed products with apparent molecular masses of 42 and 43 kDa in HSV-1-infected cells which comigrated with 42K. It is possible that the 42- and 43-kDa species found in HSV-1-infected cells correspond to the 44- to 48-kDa species reported by others (Ackermann *et al.*, 1986) using the same strain of virus (strain F) and the same monoclonal antibody (H1379). However, the relationship between these products and the closely migrating products found in the baculovirus-infected cells is not clear. The baculovirus-expressed protein is tunicamycin sensitive and thus glycosylated, as is a product of similar size found in cells infected with a vaccinia virus/gG-1 recombinant (Sullivan and Smith, 1987), while the HSV-1 product is poorly labeled with glucosamine (Ackermann *et al.*, 1986). In addition,

a similarly sized product is synthesized in the presence of tunicamycin in HSV-1 strain HFEM-infected cells (Richman *et al.*, 1986).

Biosynthesis of gG-2

A scenario for the biosynthesis of gG-2 in baculovirus-infected Sf9 cells is as follows: 107K is the primary translation product including the signal peptide. This is based on two observations. (i) It is synthesized in the absence of tunicamycin. (ii) Since no species migrating faster than 105K were detected in cells infected in the presence of tunicamycin, 107K is inferred to not be N-linked glycosylated, is therefore likely to be unaffected by tunicamycin treatment, and is probably obscured by the other species in the gel. The 105K species is the primary translation product after cleavage of its signal peptide based on its increase in abundance in the presence of tunicamycin. The tunicamycin-sensitive 118K is the cleaved primary translation product after N-linked glycosylation. Species 128K and 143K, and 110K and 120K, synthesized in the absence or presence, respectively, of tunicamycin are possibly the products of O-linked glycosylation. The tunicamycin-sensitive 34K is likely to be the result of a proteolytic degradation.

As with gG-1, there were significant differences between the biosynthesis of gG-2 in baculovirus-infected insect cells and that in HSV-2-infected mammalian cells. In both baculovirus- and HSV-2-infected cells a tunicamycin-sensitive product of 118-kDa apparent molecular mass is synthesized. In baculovirus-infected cells, it appears that this product is processed further into higher molecular mass forms, but in HSV-2-infected cells this product is cleaved to generate species of 31 kDa (31K) (Su *et al.*, 1987) and 74 kDa (74K) (Balachandran and Hutt-Fletcher, 1985) apparent molecular mass. The 74K species is subsequently O-linked glycosylated yielding a species of 105 kDa apparent molecular mass (Balachandran and Hutt-Fletcher, 1985). The 31K species is further glycosylated to a species of 34-kDa apparent molecular mass, which is efficiently secreted from infected cells, and it is not detected by the same monoclonal antibody that detects the higher mobility products (Su *et al.*, 1987). This 34-kDa apparent molecular mass HSV-2-infected cell species differs from the similarly sized product we observed in recombinant baculovirus-infected cells, in that the baculovirus product reacts with the same monoclonal antibody as do the lower mobility products.

HSV type specificity of baculovirus expressed gG

The several differences in the synthesis and processing of gG-1 and gG-2 in insect cells, relative to that observed here and by others during infections of mammalian cells with HSV, reflect differences in protein processing mechanisms between insect and mammalian cells. Despite these differences, the recombinant proteins were recognized in a HSV type-specific manner by the 10 human serum specimens tested here. To further test the use of the baculovirus expressed proteins as substrates for serologic tests we are currently testing a set of over 80 serum specimens that had previously been characterized using gG immunodot assays (Lee *et al.*, 1985, 1986; Nahmias *et al.*, 1986). Preliminary results confirm the type specificity of baculovirus-expressed gG-1 and gG-2 (Sánchez-Martínez *et al.*, unpublished data).

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